UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Establishment of a Chemical Quantitative Method for the Determination of Urinary Estrogens and its Application to a Clinical Study" submitted by Eunice Jean Chesney, B.Sc. in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The literature was reviewed with emphasis on methods for urinary estrogen determinations. The chemical quantitative methods for urinary estrogens of Brown and of Bauld were evaluated and that of Bauld was established. The method involved hot acid hydrolysis, followed by an initial ether extraction. The estriol was separated from estrone and estradiol-17 beta by means of a benzene-water partition. Purification of the estriol was done by saponification followed by partition colum chromatography. The estrone and estradiol-17 beta were separated by partition column chromatography and further purification accomplished by saponification. The final purified fractions were submitted to the Kober color reaction for color development and read on a Beckman D.U. Spectrophotometer. Allen's Correction Formula was used to obtain the corrected optical densities.

Standard graphs and maximum absorption curves for the three estrogens were determined.

Before the urines could be analyzed, column parameters were determined and the effect of temperature on partition coefficients was established. The preferred temperature was found to be 18° C.

Recovery experiments were done. Duplicate analysis showed that the method was reproducible. Special precautions in technique and the handling and cleaning of glassware were necessary.

A clinical study, to show a diurnal variation in excretion of urinary estrogens was undertaken. The subjects chosen were two young normal females, one married, and one pregnant multipara female. The results have failed to disclose evidence of a diurnal rhythm in either estrone or estradiol-17 beta urinary excretion. The results of estriol analyses were

less conclusive. Minor variation was noted in comparing a few of the day and night urinary levels. The complexity of the technique has limited the total number studied.

THE UNIVERSITY OF ALBERTA

THE ESTABLISHMENT OF A CHEMICAL QUANTITATIVE METHOD FOR THE DETERMINATION OF URINARY ESTROGENS AND ITS APPLICATION TO A CLINICAL STUDY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

by

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I. INTRODUCTION

The balance of evidence indicates at present that alterations and metabolism of steroids may be in part reflected by the quantity and nature of urinary metabolites. This is the reason for the estimation of urinary metabolites of the principal estrogen. Accumulating evidence favors the view that this principal estrogen, elaborated by the ovary, placenta, adrenal and testis may be identical with estradio-17 beta. Only a small portion of the estradiol-17 beta synthesized is excreted in the urine as such. The majority is excreted as reduced derivatives.

To date, six estrogens have been isolated from human urine. These are estrone, estradiol-17 beta, estriol, 16 epiestriol, 16-alpha-hydroxyestrone and 2-methoxyestrone. It has been suggested (1) that the metabolic precursor of both estriol and 16-epiestriol might be 16-oxo-estradiol-17 beta. The presence of this latter estrogen is held in question because in alkaline solutions at room temperature 16-alpha-hydroxyestrone undergoes rearrangement to 16-oxoestradiol-17 beta which would be produced as an artifact during the separation of the phenolic fraction by partition between ether and alkali. (2). Investigators (3,4,5) have recently shown an enzyme system in human placenta which catalyzes the interconversion of estrone and estradiol.

A hypothetical scheme for the interrelationship of the first five estrogens in urine is given.

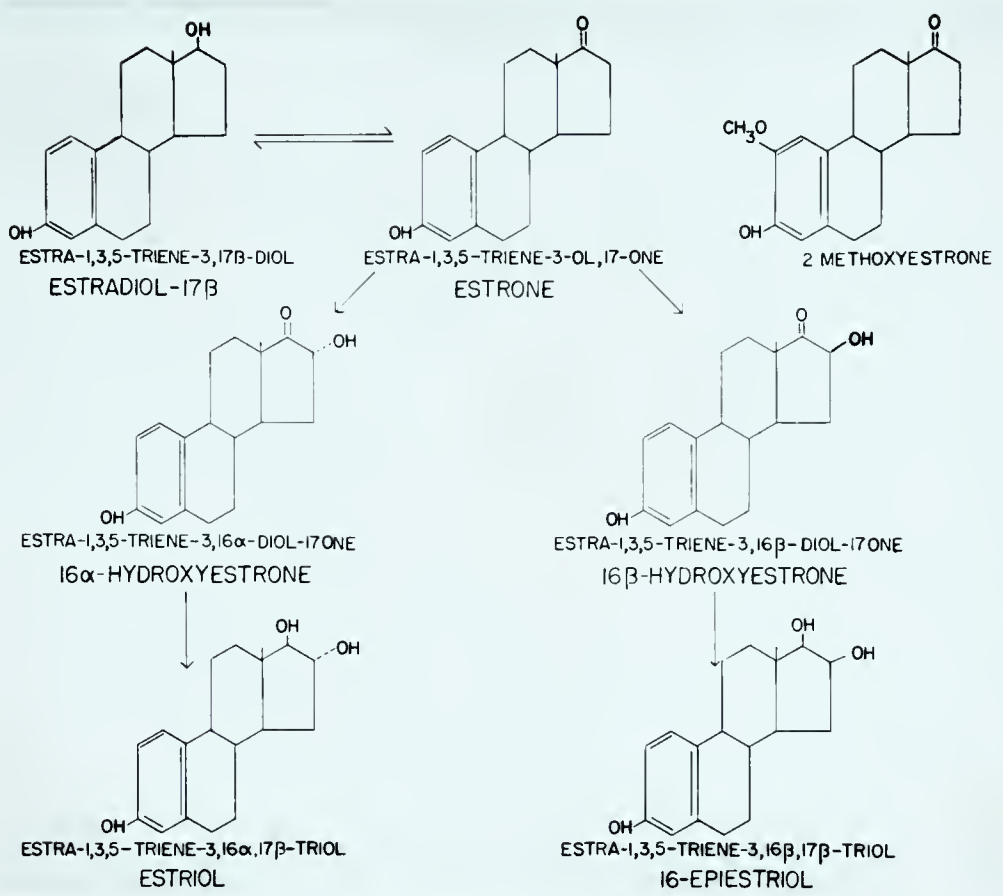
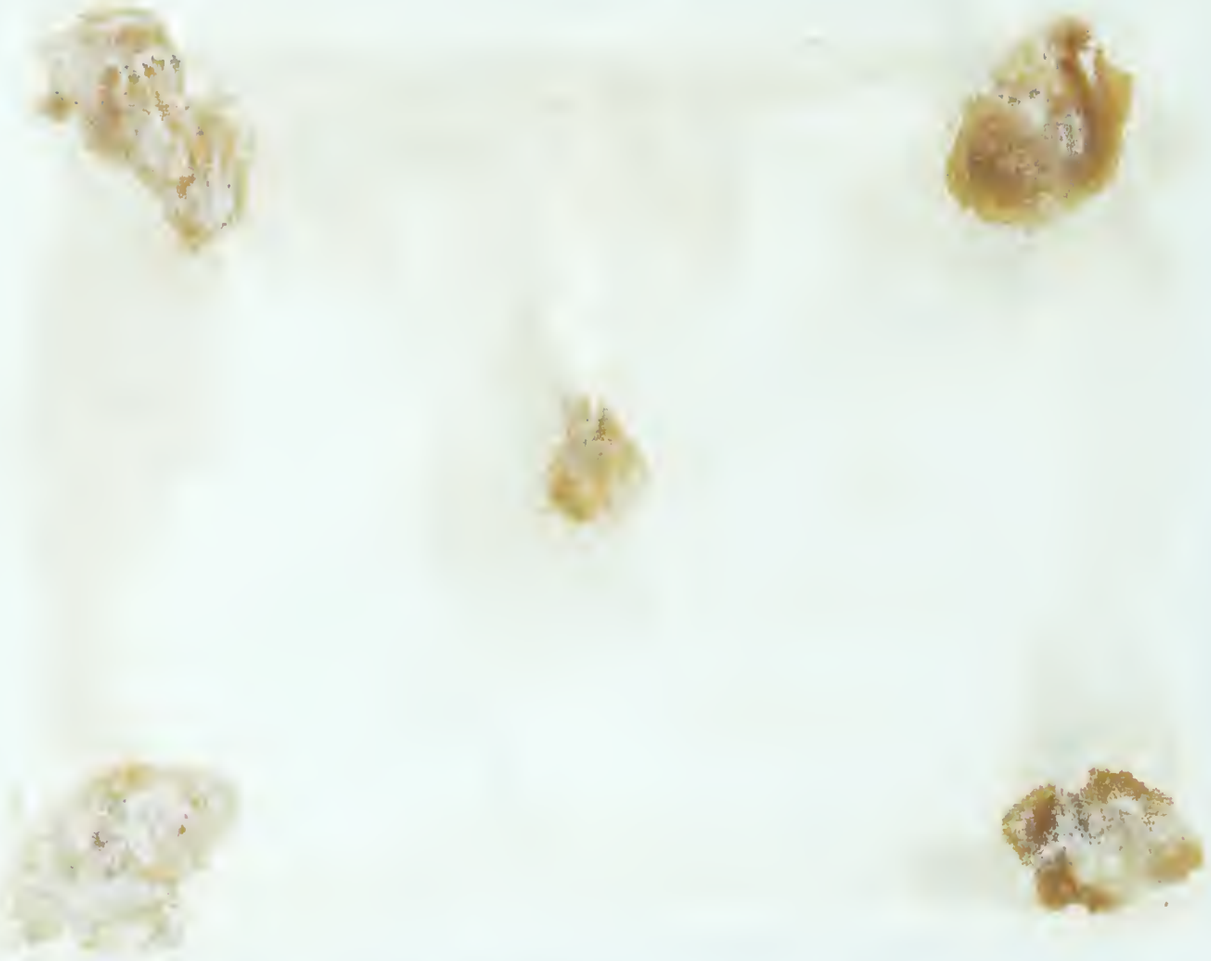


Fig. 1. Possible Interrelationship of Five Urinary Estrogens.

Although methods have been reported for determining all six of the known urinary estrogens (6) the majority of methods employed determine only estrone, estriol and estradiol-17 beta. The three other estrogens are normally found in such extremely small quantities. They require specific hydrolysis and purification steps which makes the procedure longer and more involved



A. METHODS SUGGESTED FOR THE ESTIMATION OF URINARY ESTROGENS: (7)

(1) Biological Methods
  (a) Vaginal cornification (8)
  (b) Increase in uterine weight (9,10,11)

(2) Physical Methods
  (a) Polarography
  (b) Ultraviolet absorption spectrophotometry
  (c) Infra-red absorption
  (d) Radio-active tracer-technique

(3) Biochemical Methods
  (a) Enzymic reactions

(4) Chemical Methods
  (a) Fluorimetry
  (b) Colorimetry

(1) ANALYSIS OF THE BIOLOGICAL METHODS:

The disadvantages of the biological methods are outlined:(12)

(a) The estimation of estrogenic potency depends largely on the method of assay adopted. Minor variations in techniques such as alteration of solvent or of spacing of injections influence the final result.

(b) Impure urinary extracts have been shown to contain a mixture of estrogens which differ widely in their biological activity. Such extracts therefore could not be assayed in terms of the international standard using estrone. To separate the estrogens prior to bio-assay and subsequently express the results in terms of the weights of pure crystalline substances would be laborious, time consuming and expensive. The number of animals required for a necessary degree of precision would be enormous.

(c) Human urine concentrates may contain substances which might enhance or perhaps suppress the effect of estrogens on the target organs. Their presence in urinary extracts would likely be found to vary according to the method of extraction used.

Bio-assay results should be used as a standard of reference in order to prove that estrogenic material is in fact being measured by other methods. (13)

(2) PHYSICAL AND BIOCHEMICAL METHODS:

Among the physical methods ultraviolet absorption photometry (14) and infra-red spectrophometry (15) have been reported to require highly purified extracts and are not sufficiently sensitive for most urinary assays. Polarographic methods (16) do not measure all types of estrogens. Radioactive tracer techniques (17) and biochemical methods based on enzymic reactions (5,18) have shown great promise but have not been adequately evaluated to be applied to clinical investigation.

(3) CHEMICAL METHODS:

Chemical methods therefore appear to be the methods of choice. The fluorimetric or colorimetric techniques depend upon the findings of Kober (19). Kober reported that estrone when heated with concentrated H_2SO_4 produced a green fluorescing orange colored solution that turned pink on dilution with water. The addition of phenol decreased the fluorescence and thereby simplified the solution for colorimetry. Kober's method, using a three stage dilution was unstable. It was first applied to the estimation of urinary estrone and estriol in 1934 (20) with the elimination of the third dilution. Other workers (21) using the three stage dilution but maintaining rigid control over time and temperature found the reaction still unstable. The instability of the reaction was investigated (22) with respect to optimum conditions of time, temperature, acid concentration, and the necessity of the presence of a reducing agent. When the reaction was applied to urinary estrogens there was a depression in the amount of color developed due to contamination by solvent and urinary residues. Therefore the reactions were re-investigated. (23) The final modified reagent i.e. aqueous H_2SO_4 hydroquinone with trace amounts of nitrate and quinone added and the addition of hydroquinone to the reaction tube before a first and second boiling, diluting to 60% concentration, gave a color reaction which was stable for several hours.

The specific groups involved in the Kober reaction have been investigated. (24) They were found to be a phenolic or phenolic ether group at position 3 and an intact ring D oxygenated at position 17. Oxygenation at position 16 or at both the 16th and 17th positions gave no color. Hence the specificity of the Kober reactions for estrogens was established.

The estrogen fractions frequently have been found to contain non-estrogenic pigments. The pink color of the estrogen reaction product is superimposed on the non-specific color due to these chromogenic impurities. Methods have been used which decolorize the estrogen product and the true estrogen value is determined by difference. (20,25) The colorimetric methods of Brown (26) and Bauld (27) use the Allen's Correction Formula. (28) The corrected optical densities were calculated from measurements taken at the wavelength at the absorption maximum for the specific substance being involved and at two wavelengths equi distant from this maximum. The equations outlined were found to be valid if the three optical densities of the non-specific chromogens lie on a straight line. This has been shown to be true in results reported for normal urines and some pathological urines. (27) It has been suggested that the chromogens present in other pathological urines may not demonstrate linearity. (22)

The Allen Correction Equation:

$$CDX_x = OD_x - \tfrac{1}{2}(OD_a + OD_b)$$

OD_x being the observed optical density at wavelength at the absorption maximum.

OD_a and OD_b being the observed optical densities at wavelengths " a & b " which are equidistant from x.

CDX_x being the calculated optical density due to substance X having an absorption maximum at wavelength x.

Fluorimetric methods have been used by many workers. (29,30,31, 32,33,34,35,36) Optimum conditions have been reported (22,37). Considerable discrepancy was found in the accuracy of their findings. It has been suggested (38) that the disagreement of various workers using the fluorimetric technique was due to differences in acids, and their concentrations, and equipment. The type of estrogen present affected the fluorescence reaction. (39) 2-methoxyestrone does not fluoresce in H_2SO_4 and would therefore be missed. Urine extracts following countercurrent distribution were noted to contain non-estrogenic substances which fluoresce in H_2SO_4. Therefore although fluorimetric methods were found to be more sensitive than colorimetric methods, they were less specific. The use of the fluorimetric method awaits the perfection of methods to eliminate these non-estrogenic fluorescing substances. In the meantime, colorimetric methods are considered to be the most satisfactory.

The specificity and accuracy of both the fluorimetric and colorimetric methods would appear to be governed by the methods of hydrolysis, extraction, and purification of the estrogens.

It has been shown that urinary estrogens are excreted as conjugates of glucuronic or sulfuric acids. In 1936 this fact was proven when crystalline sodium estriol glucosiduronate was isolated and identified. (40) These same workers showed that the conjugation occurred on position 16 or 17 of the steroid nucleus. Since that time it has been shown that normal humans excrete small amounts of estrogens conjugated as sulfates. (41,42) Katzman, et al. (42) demonstrated by means of enzymes and acid hydrolysis, that estriol was conjugated as both the glucosiduronate and sulfate, mostly as the former. The majority of the estrone-estradiol fraction was excreted as glucosiduronate with a small amount as the sulfate. Because urinary estrogens were found to

be excreted in this chemical fashion and not in the free form, hydrolysis of urines was performed for two reasons. The chemical nature of all the conjugated forms was not known so that all methods of assay have been developed using the readily available free estrogens as standard material. The final reason was that extraction with organic solvents remained a convenient method for the separation of estrogens from the highly water soluble compounds that constitute the bulk of urinary solutes.

The two main methods of hydrolysis that have been used are hot acid hydrolysis and enzyme hydrolysis. Two principal procedures for hot acid hydrolysis have been developed. The first of these was autoclaving (20,31) which was replaced by refluxing for convenience. (25) Refluxing became the accepted method for hydrolysis. Certain discrepancies reported resulted from variation in the type and concentration of the acid used and the time allowed for hydrolysis. The duration of boiling required for complete hydrolysis was found to depend on acid concentration. Several workers have shown (37,42,43) that ten minute refluxing in 15 volumes % hydrochloric acid was too short a time. This technique had previously been suggested. (44) Evidence accumulated (26,45) showed that the addition of 15 volumes % of concentrated hydrochloric acid to boiling urine and refluxing for sixty minutes were the optimum conditions for satisfactory hydrolysis. It appeared generally agreed that this compromise permitted maximum yield of the three estrogens from the majority of urine.

Considerable evidence accumulated showed that the estrogens in pure solution were stable to boiling with acids in the concentrations required for the hydrolysis of their conjugates. (46) General agreement appeared to be present that losses did occur during acid hydrolysis of urines. This loss appeared due to the combined effect of a normal constituent of urine

and boiling with acid. It was found to be preventable by diluting the urine with water before hydrolysis where the amounts of estrogen present were high. (46) The nature of the urinary constituent which caused destruction is not known. The loss of estrone and estriol during acid hydrolysis was proportional to the total amount of estrogen present. The loss of estradiol-17 beta was found to be proportional to the amount present when the concentration was high but might be complete when the concentration was low. This suggested two destructive processes operating in the instance of estradiol-17 beta. (46)

Enzyme hydrolysis appeared to be more advantageous because of its reduced rate of estrogen destruction. (46) Further, some of the newly discovered estrogens such as 16-hydroxy-estrone, were found to be labile in hot acids. The disadvantages of enzyme hydrolysis were the length of time required, the expense of enzyme preparation, the sensitivity to inhibition and to cause emulsions in the extraction prodedure. There is evidence, however, that the products of enzyme hydrolysis show an increased number of impurities in the estriol fractions while the estrone-estradiol-17 beta fractions were reported to contain less impurities. (46). The type of enzyme preparation used (42) and its source (47) were found to have an influence on the degree of contamination encountered. Methods of enzyme hydrolysis have been used to determine 2-methoxyestrone, estrone, ketolic estrogens, estradiol-17 beta, 16-epiestriol and estriol. (6)

The chief difficulty in the chemical estimation of urinary estrogens has been found to be the presence of extraneous material and pigments which mar the final reaction product for both colorimetry and fluorimetry. For this reason, the hydrolyzate has been submitted to rigorous purification prodedures. The estrogens accompanied by other acid materials were removed from the hydrolyzate by ether extractions. The

methods for the subsequent removal of the acidic material have varied. When 10% sodium carbonate was used, the estriol level was reduced. (20) When saturated sodium bicarbonate was used, the extract was not as pure, (31,37) non-estrogenic fluorescing material being present. Estriol and estradiol could not be found in urine of a non-pregnant woman in the pre-ovulatory phase of menstruation even after countercurrent distribution suggesting the removal of these estrogens by the bicarbonate. It has been demonstrated (26) that the distribution of estriol between ether and weak alkali depended more on the ionic strength than on the pH of the aqueous phase. Using a carbonate buffer of pH 10.5 the acid fraction was removed without loss of estriol. Further removal of pigments was accomplished by adding sodium hydroxide, followed immediately by 1 M sodium bicarbonate to increase the ionic concentration. A further wash with bicarbonate to remove the alkali followed. These steps in purification were used in both the commonly used colorimetric methods. (26,27) Further purificiation was achieved by saponification. (27) This has been incorporated into the method of Brown. (48) Here it was reported to eliminate the effect of administered substances such as cortisone, stilboestrol, and the substance present when the urine was hydrolyzed with enzymes of Patella vulgata. These had been found previously to interfere with the color reactions.

The extracts must be further purified and separated. Several methods have been used.

Adsorption chromatography on alumina columns was first used by Stimmel. (49)Difficulty was reported in eluting estriol from the columns. Defects in the method have been reported. Variations in elution with different batches of alumina, tailing and displacement effects due to impurities were pointed out. (37) When pure solutions were used, the

three estrogens could be separated. Successful use of adsorption chromatography was found to depend upon precise standardization of equipment, reagents and adsorbent. Brown, who used deactivated alumina, standarized with pure estrogens, formed the methyl ethers of the estrogens prior to subjecting them to adsorption chromatography. (26) His method has also been used to determine the estrogen levels in meconium of newborn boys. (50)

Ion exchange chromatography has also been used, followed by separation by means of paper chromatography. (51) Although paper chromatography has been used for many biochemical assays, it is not considered as accurate in quantitative estimations of urinary estrogens. Paper has been reported to contain " dirt " which was found to interfere with spectrophotometric determinations. (52) The standardization and use of standard estrogens simultaneously is a part of paper chromatographic technique.

Gradient elution chromatography has also been used to purify and separate urinary (33) and plasma estrogens. (34) In addition it has been used for the separation of radio-active labelled estrogens. (53)

Countercurrent distribution has been used by several workers. (31,32,54,55,56) The advantages appeared to be in the realm of adequate separation and characterization by partition coefficients and quantitative estimation of each estrogen or contaminant in the sample analysed. The disadvantage consisted of its cumbersome nature, and time consuming characteristics particularly when several distributions were necessary for purification purposes, and its expense.

Column Partition chromatography consisted of separation of solutes by distribution between two liquid phases on a chromatographic column. This method of purification and separation has been used by many

workers. (15,27,35,57) The differences from worker to worker consisted chiefly in the solvent systems used. In the method of Bauld, (27) estriol and other hydrophilic compounds were removed from the estrone-estradiol fraction prior to chromatography by partitioning them between water and benzene. This fraction, estriol was separated from other hydrophilic substances by a partition chromatogram using a particular solvent system. The factors influencing the elution pattern of both chromatograms have been investigated, i.e. temperature, sodium hydroxide concentration, etc. (58)

Experiments have been reported demonstrating how closely an analytical result approaches a true value, (accuracy), the reproducibility of results (precision), the least amount of the substance analyzed which would be distinguishable from zero (sensitivity) and whether the method of analysis measures that compound which it is supposed to measure and nothing else (specificity). (48)

In assessing the existing chemical methods, the colorimetric methods of Brown (26) and Bauld (27) would appear to fit these criteria. These two colorimetric methods have been compared by their inventors in parallel assay and the results obtained with them reported to agree quite favorably. It has been suggested (7) that with recent instrumental improvements, flurometric methods may become more satisfactory.

The use of the spectrophotometric correction i.e. Allen's Equation, (28) has been tested by countercurrent distribution studies. (54) It has been found to be valid using normal and some pathological urines. The overall specificity has been tested (48,55,56) and the results have confirmed the method to be specific for estrone, estriol and estradiol-17 beta. The sensitivity of the methods is reported to be approximately 1.5

micrograms per twenty-four hours. (48) Using recovery experiments in which known amounts of estrogens were added to the hydrolyzed urine, the accuracy of the method has been studied. (27,48)

With the available data suggesting that the colorimetric methods are the most appropriate for the estimation of estrone, estriol and estradiol-17 beta, considering the availability of existing equipment, the method of Bauld was chosen for establishment in this area.

II. METHODS AND MATERIALS

(A) MATERIALS:

Diethyl ether - Reagent grade. Peroxides were removed by shaking for three minutes with approximately 0.3 M Fe SO_4 in 0.4 N H_2SO_4 (100 ml/liter) washing three times with distilled water (100 ml/liter) and distilling within 6 hours of use.

Benzene - ANALAR - thiophene-free, distilled from 1/20 volume liquid paraffin and redistilled.

Methanol - ANALAR - acetone-free, distilled from 1/20 volume liquid paraffin and redistilled.

Ethylene dichloride. ANALAR - This was poured through a column (4 feet x 1 inch) of silica gel (28 - 200 mesh) at approximately 10 ml/minute. (The silica gel was discarded after 2 liters). The purified solvent was distilled within 24 hours of use.

Ethanol - Allowed to stand over m-phenylenediamine (10 grams/2 liters) for at least one week, then distilled and redistilled.

Concentrated Carbonate Buffer (approximately pH 10.5) - 150 mls. 10 N NaOH, 150 mls. H_2O 2 liters 1 M Sodium Bicarbonate.

Celite 535 (Johns Manville & Co. Ltd.) The crude Celite was heated at 400° C for four hours, stirred with concentrated HCL to a thick slurry and left overnight. It was then washed with distilled water by decanting until the washings were free of chloride ($AgNO_3$ test) and iron (KCNS test) and free of acid (pH equalling that of the distilled water). It was dried at 110° C for 48 hours and cooled in a vacuum desiccator over Phosphorus Pentoxide. Celite for the benzene - NaOH column was kept over Phosphorus Pentoxide until used while the celite for the ethylene dichloride-aqueous methanol columns were stored in a tightly-fitting stoppered container. Where possible the celite for both columns was kept in an oven at 110° C. The amount

required for chromatography was removed the night before use and placed in a desiccator.

Color Reagents - The preparation of the reagent for estradiol-17 beta, concentrated H_2SO_4 (ANALAR) was diluted to 60% (v/v). To a 1000 ml. graduate cylinder, 600 mls. of concentrated H_2SO_4 were added. 440 mls. of distilled H_2O were poured into a 2 liter round bottomed flask. The acid was poured slowly from the cylinder into this and cooled with tap water. When the flask was brought to room temperature the contents were poured into the cylinder used to measure the acid. The volume was made up to 1000 ml. using distilled water and the contents poured back into the 2 liter flask while cooling under tap water. This operation was repeated until 1000 ml. of 60% (v/v) of H_2SO_4 at room temperature was obtained. 10 mgm. sodium nitrate (0.5 ml. of a 2% solution), 20 mg. of redistilled quinone were added to the acid. The flask was warmed under tap water until the solution turned yellow-green in color, immediately 20 gms. hydroquinone were added. The hydroquinone was dissolved by shaking and heating in a boiling water bath. When hydroquinone was dissolved, heating was continued for 45 minutes. Following heating it was placed in a dark cupboard for a week. It was then filtered through a fine sintered glass and stored in dark bottles. The final solution was a definite pink color and non-opalescent.

Estrone and estriol reagents were prepared in exactly the same fashion except that 66% (v/v) and 76% (v/v) respectively of H_2SO_4 was used. The final solution for estrone was yellow with a trace of pink and for estriol, yellow without evidence of pink color.

All reagents used in this procedure were ANALAR.

FLOW SHEET OF PROCEDURE

(A) URINE Hydrolyzed with HCL

| Extraction with Ether

ETHER EXTRACT

(B) Washed with conc. CO_3^{2-} Soln, pH 10.5

(C) SHAKEN with 2 N NaOH

(D) M - $NaHCO_3$ added, shaken, combined aqueous Extract discarded

(E) Washed with M - $NaHCO_3$ and water

ETHER Evaporated

Residue

(F) Dissolved in ethanol : Benzene | Water

Aqueous Extract

(G) NaOH

(H) Reflux 30 minutes

(I) Extracted with ETHER (discarded)

(J) Aqueous to pH 9.3

(K) Extracted with ether, washed with $NaHCO_3$ and water

Ether evaporated

Impure estriol fraction

(L) PARTITION CHROMATOGRAM

ESTRIOL FRACTION

Benzene

Evaporated to dryness

ESTRONE - ESTRADIOL - NEUTRAL FRACTION

(M) PARTITION CHROMATOGRAM

Neutral Fraction

Impure Estradiol

IMPURE ESTRONE

(N) Saponified 30 min.

Acidified

Estracted with Benzene

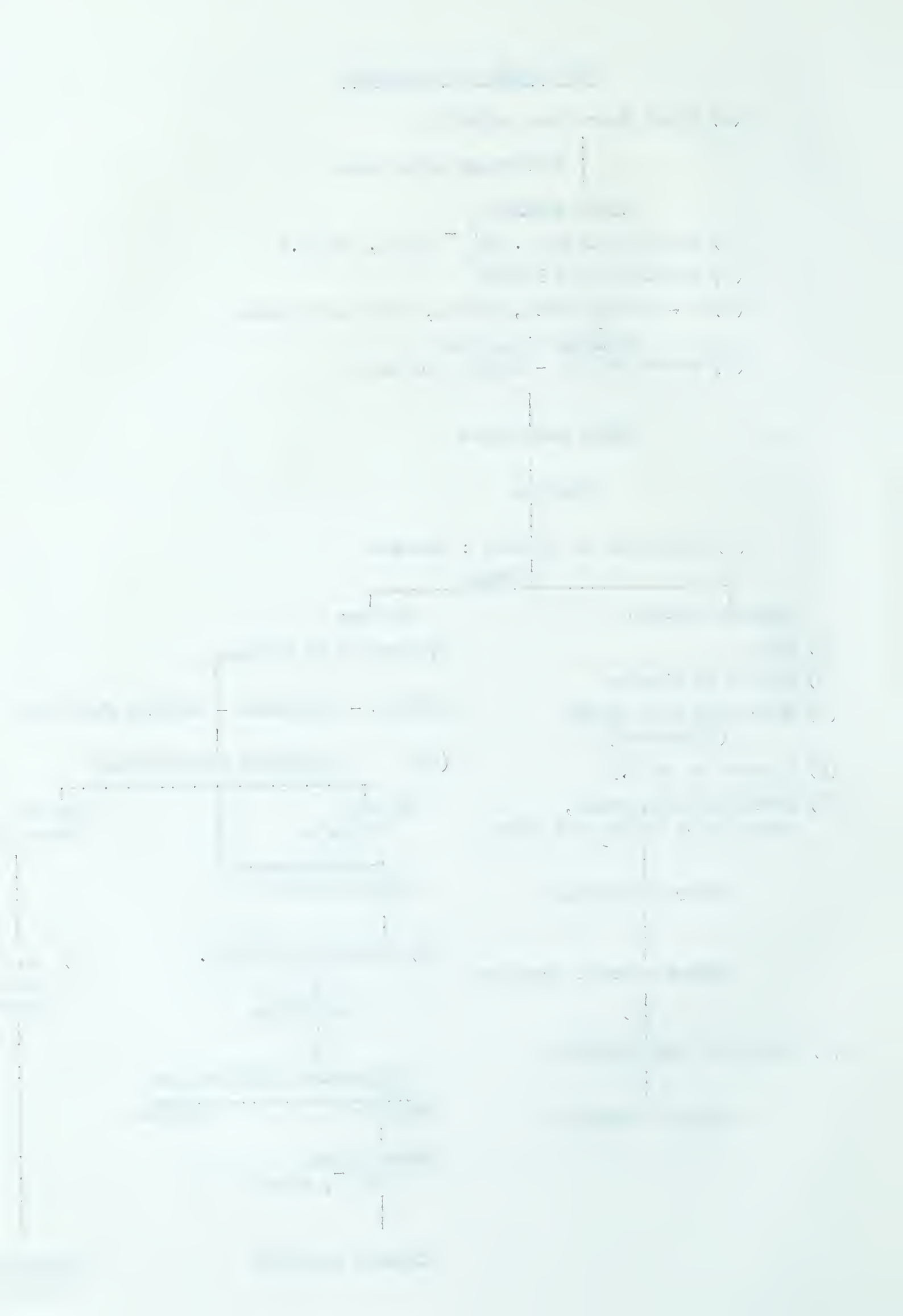
Benzene | Aqueous

Washed with CO_3^{2-}, water

ESTRONE FRACTION

(O) As for impure Estrone

ESTRADIOL FRACTION



(B) METHOD:

(1) Urine Collection: Twenty-four hour urine samples were collected in two liter glass stoppered bottles or polyethylene bottles. During the collection the urine was kept as cold as possible with the glass stopper resting in a funnel in the top of the bottle. The samples were stored under refrigeration at 4° C. if determinations were accomplished within forty-eight hours. They were otherwise placed in a deepfreeze. (59) The bottles used for urine collection were thoroughly rinsed with tap water followed by filling with chromic acid and standing for twenty-four hours. They were then emptied and thoroughly rinsed. Crude ethanol was added with exposure of all parts for at least five minutes. Cleaning in this manner was found necessary for elimination of bacterial contamination and variations in the quantities of estrogen present in varying urines.

(2) Hydrolysis of Urine: The urine volume was noted and then diluted to 2500 ml . with distilled water. Two 500 ml. Aliquots were measured into round bottomed liter flasks. A 4 mm. glass bead was added to each flask utilizing forceps and the flasks attached to condensers marked hydrolysis. The urine was brought to a boil under reflux. 75 ml. of concentrated hydrochloric acid was then added slowly through the top of the condenser and the specimen permitted to reflux for sixty minutes. It was then cooled under the cold water tap. The condensers were washed down with dilute sodium hydroxide and distilled water to eliminate the aromatic substances which might contaminate future specimens.

(3) Initial Extraction: The cooled hydrolyzates were added to 1 liter separatory funnels, the stopcocks having been lubricated by the addition of distilled water. Extraction was carried out with ether four times. The first using 150 ml. and the following three with 125 ml. volumes. The extracts were collected in 500 ml. conical flasks. Following the fourth

extraction, the 1 liter round bottomed flasks were emptied, washed and drained for future use.

(4) Removal of the Acid Fraction: The ether extractions were combined in a separatory funnel. It was subjected to vigorous shaking. A small amount of the aqueous phase was permitted to run out along with the bulk of the interfacial residues. 100 ml. of carbonate buffer was added to each conical flask permitting it to run down the sides. This was done to facilitate subsequent cleaning of the conical flasks. The carbonate was transferred to the appropriate separatory funnel and shaken thirty times. The buffer was removed and the ether then shaken with 25 ml. of 2N NaOH. At this point the bulk of estriol was in the small volume of aqueous phase. It was therefore essential that no leakage occurred from the stopcock. Without removing the aqueous phase, 100 ml. of 1 M Na bicarbonate was added to the separatory funnel and shaken 100 times. This was done to bring the pH of the solution to approximately 10 and to re-extract the estrogens into the ether phase.

After separation, the aqueous phase was then discarded and the ether washed with 25 ml. of 1 M sodium bicarbonate. Following separation the lower phase was run out completely. Distilled water was used to wash the stopper permitting the water to run back into separatory funnels. The inlet and walls of the separatory funnel were carefully washed. The volume of water was reduced to 10 to 15 ml. and the remainder shaken thirty times. The aqueous phase was then removed completely and the ether phase decanted quantitatively to the rinsed and drained 1 liter round bottomed flask. A glass bead was added using forceps and the ether distilled off into an ether recovery bottle. Ether vapor was removed from each flask while it was still warm with a gentle current of filtered air.

(5) Benzene - Water Partition: The residues from ether distillation were dissolved in ethanol (1.5 ml.) and transferred to 250 ml. separatory funnels with 25 ml. of benzene added in portions permitting washing of both the flasks and beads. The flasks were rinsed with 25 ml. of water which was then added to the separatory funnels. This was shaken thirty times. Because of the high interfacial tension of the benzene and water, persistent emulsions were easily obtained at this stage with excessively vigorous shaking. Shaking was therefore vigorous enough to insure equilibration but without causing emulsification. The aqueous layer was run into 250 ml. round bottomed flasks. Washing of the benzene phase was repeated with an additional 25 ml. of water and twice with 12.5 ml. aliquots of water. The four washings were combined in 250 ml. round bottomed flasks and were assumed to contain the hydrophilic fraction. This was reported to be precise partition and the volumes of benzene and water expected to be measured most accurately. The benzene layers containing the estrone-estradiol fraction were then transferred quantitatively with additional benzene to separate 250 ml. round bottomed flasks and evaporated under vacuum after the addition of glass beads.

The hydrophilic fractions were made alkaline with 10 N sodium hydroxide (7.5 ml.) and refluxed for thirty minutes following the addition of glass beads. Following refluxing, they were cooled under tap water and transferred to 250 ml. separatory funnels and extracted with 100 ml. of ether , which was then discarded. The alkaline solutions were returned to the same separatory funnels and brought to a pH of 9.3 to 9.5 by adding CO_2 from a manifold through 1 mm. capillary tubes. An additional separatory funnel containing 75 mls. of water, 7.5 mls. of 10 N sodium hydroxide and 1 to 2 drops of thymolphthalein served as an indicator of completion. With

loss of color in the indicator funnel, the CO_2 addition was stopped. The pH of the other funnels was evaluated using short range pH paper. On completion, the capillary tubes were disconnected and washed with ether and then withdrawn. The partially neutralized solutions were extracted with ether four times using 40 ml. aliquots. The extracts were collected in 250 ml. conical flasks and returned to the separatory funnels following completion of the extraction. The ether was then washed with 5 ml. of M sodium bicarbonate which was discarded. The funnel, stopper, and top of separatory funnels were washed in water. The water was permitted to run off. 5 ml. of water were then added and shaken. The stopper was washed permitting the water to run into the separatory funnel and then the water was permitted to run out. The separatory funnel was again washed with water taking care to include the neck and walls. This water was run off without shaking. It was important that the extract at this stage be neutral as the next stage was partition chromatography where the pattern of elution might be modified by the presence of bicarbonate. The extracts were then transferred quantitatively to 500 ml. round bottomed flasks and distilled to dryness.

(6) Chromatography

(a) Equipment: The chromatography tubes were glass 30 cm. long and manufactured from selected uniform bore tubing 1 cm. in diameter. The top of the columns consisted of a 14/35 standard tapered joints and the lower ends sealed, flattened and perforated with approximately 8 pinholes. The inner surfaces of these perforated ends had to be perfectly flat otherwise the celite in this area would be packed too lightly and could be washed out by mobile phase during chromatography.

Packing was accomplished with a plunger made of a perforated brass disc attached by means of a stirrup to a handle of stainless steel.

The handle was approximately 60 cm. long. The diameter of the disc was only slightly less than the internal diameter of the chromatography tube. The perforations in the disc were large enough to permit the plunger to pass through a celite slurry when moved quickly for purposes of homogenization but small enough to permit the homogenized slurry to be packed down when the plunger was moved in a slow fashion. Perforations of standard wire gauge 22 (0.71 mm.) were suitable when the interfacial tension between the two liquid phases was low, such as in the case of aqueous alcohol as stationary phase. 19 gauge (1.02 mm.) perforations were used where benzene-aqueous solution systems with a high interfacial tension were used.

The reservoirs for mobile phase consisted of 125 ml. separatory funnels with delivery stems drawn out and bent in such a way as to permit the solvent to run down the sides of the chromatography tubes when in position. A 14/20 cone was sealed to the stem of each funnel above the bend. No lubricant was used on stopcocks.

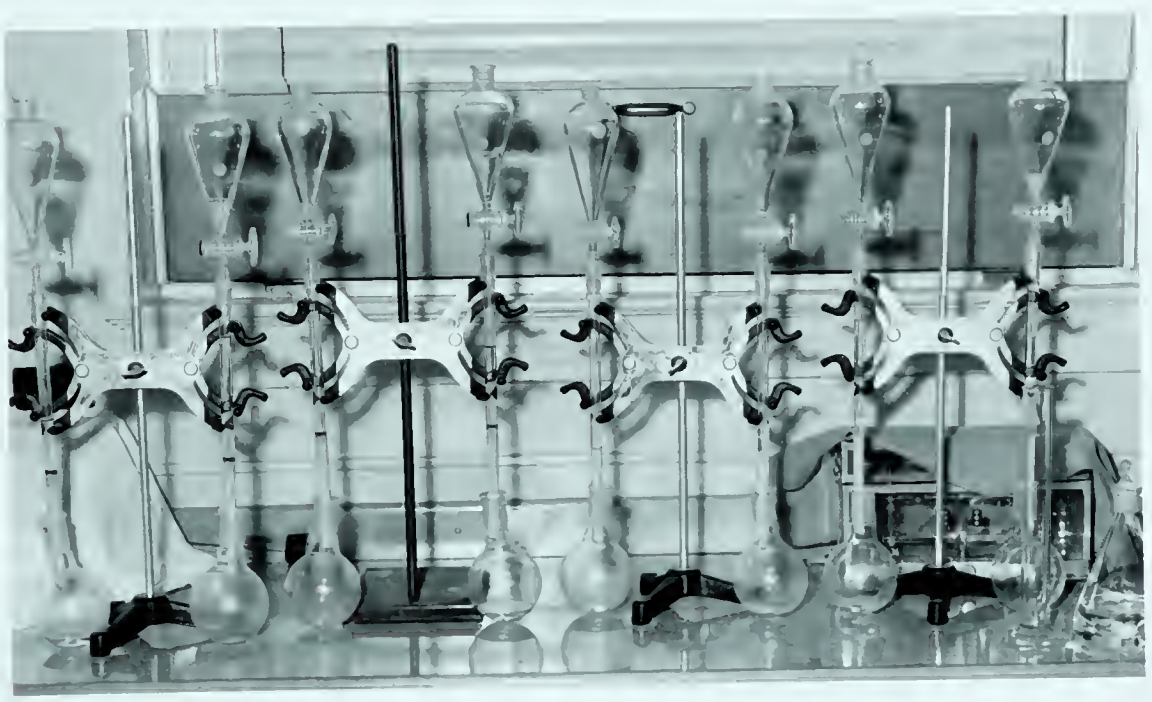
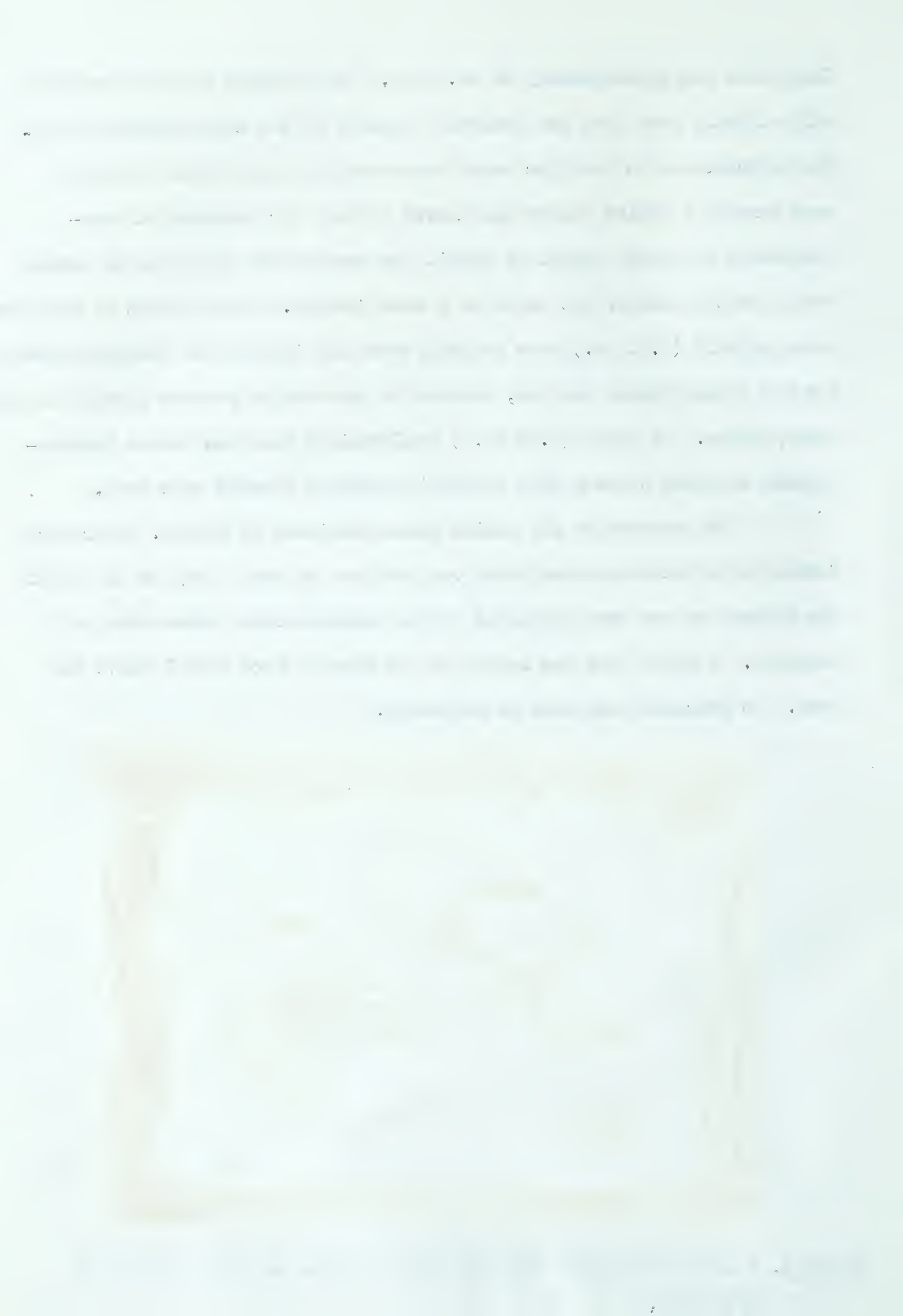


Figure 2. A set of Partition Columns being used for the assay of Urinary Estrogens.



(b) General Description of Chromatography: In the preparation of the partition chromatogram, two immissible solvents were shaken together and allowed to separate. The more polar of the two, (usually an aqueous solution) became the stationary phase. of the chromatogram and the less polar, (organic) became the mobile phase. The separated stationary phase was mixed in known proportions with the supporting medium, in this case celite. The stationary phase would then be strongly adsorbed within the micelles of the celite. Excess mobile phase was added to form a wet slurry which was then packed into the glass chromatography column. Care was taken to avoid the presence of air bubbles. The mixture to be partitioned was added to the top of the column in a minimum volume of mobile phase. Additional mobile phase was then added and allowed to percolate through the column. As this mixture proceeded down the column, continuous distribution occurred between the mobile phase and the stationary phase. The solute with the least solubility in the stationary phase, that with the lowest partition coefficient, was not retained on the column as much as the solute with the greatest solubility in the stationary phase. Therefore by collecting effluent from the column in small fractions it was possible to separate the various solutes as they arrived at the bottom of the column in solution in mobile phase.

The night before chromatography, an adequate amount of dried celite was removed from an oven and placed in a desiccator containing phosphorus pentoxide and brought to the temperature of the chromatography room. All solvents used in chromatography were also permitted to equilibrate at the temperature of the chromatography room.

i. Solvent-Phase-Preparation: 210 ml. of purified methyl alcohol was added to a graduated cylinder and diluted with water to 300 ml. and mixed. This was added to a special separatory funnel. When the temperature of the

solution was equilibrated to the temperature of the chromatography room, 300 ml. of ethylene dichloride were measured in the same cylinder, added to the separatory funnel and the two shaken fifty times.

(b) For Estrone Chromatography: 300 ml. of 0.8 N sodium hydroxide were added to a separatory funnel. This sodium hydroxide was checked weekly by titration. 300 ml. of purified benzene were added. Following temperature equilibration, they were shaken fifty times.

(c) For Estradiol Chromatography: 195 ml. of ethylene dichloride and 65 ml. of benzene were added to a glass-stoppered flask and mixed thoroughly. The solvents were allowed to equilibrate overnight in the temperature controlled room.

ii. Preparation of Chromatograms:

(a) Chromatography of Estriol Fractions: 20 grams of celite were weighed out in a 300 ml. beaker. The lower phase of the estriol solvents was transferred to a 500 ml. glass-stoppered flask, clearly labelled "estriol mobile phase." The upper stationary phase was carefully transferred from the top of the separatory funnel to a 500 ml. glass-stoppered flask, cleared labelled, "estriol stationary phase." 20 ml. of stationary phase were pipetted into the beaker containing the celite (1 ml./gm. of celite) and mixed thoroughly with a glass rod for three minutes. Mobile phase was added until a stiff slurry was formed. The lower end of the chromatographic column was placed in a test tube supported on a rubber stopper and clamped loosely in a vertical position. 10 ml. of mobile phase were poured into the column. The few grams of celite slurry were then added. With the plunger made of 0.71 mm. perforations the plug of slurry was pushed down the column into the mobile phase and homogenized with rapid vertical movements. Following homogenization the celite was packed down using slow vertical motions of

the plunger. The column was rotated to insure even packing. The plunger was held between the thumb and the index finger and pressed down as firmly as possible on the surface of the packed celite. It was attempted to have a reproducible pressure with each subsequent layer of celite. Slurry was again added to the column in batches sufficient to form one to two cm. of height on the column, homogenized in the mobile phase and packed into segments of 1 to 2 mm. until a 10 cm. length of column was obtained. Close observation for a flat surface on the top of the column was made and then the excess mobile phase poured off along with loose celite. The top of the column was washed with mobile phase to remove any excess celite. 1 ml. of estriol mobile phase was added to the 500 ml. round bottomed flask containing the estriol residue. The flask was rotated to dissolve the extract. The extract was then transferred using a 1 ml. volumetric pipette to the top of the celite column holding the pipette against the wall of chromatogram a few millimeters above the surface of the column. The transfers were made twice more making a total of three transfers from each flask. The eluate for these 3 ml. was collected in a beaker and discarded. As the final millimeter of extract disappeared into the top of the celite column, a separatory funnel containing 30 ml. of mobile estriol phase was attached to the column. A few milliliters of the mobile phase were permitted to run down the side walls of the tube in order to provide a cushion for the remaining mobile phase. The next 10 ml. of eluate were collected in a graduated cylinder, the following eluates collected in 500 ml. round bottomed flasks. The solvent was removed the following morning <u>in vacuo</u> after the addition of a glass bead.

(b) <u>Chromatography of estrone-estradiol fractions:</u> Columns were packed using the same procedure for column packing as outlined, for the estriol columns.

These columns were packed to 12 cm. in length. The slurry was prepared from 25 grams of dried celite with 20 ml. of stationary phase (0.8 N sodium hydroxide, lower phase.) This amounted to 0.8 ml. per gram of celite. The slurry was then stirred for three minutes with a glass rod and excess mobile phase was added to form a stiff slurry. The plunger with the 1.02 mm. perforations was used. One ml. of mobile phase (benzene upper phase) was added to each flask. The flasks were rotated using the warmth of the hand to dissolve the extract. The extract was transferred to the top of the celite column using precautions outlined for estriol chromatography. The transfers were repeated twice making a total of three transfers per flask. The eluate from the column for this 3 ml. was collected in a beaker and discarded. When the final ml. of extract disappeared into the celite column, a separatory funnel containing 40 ml. of benzene mobile phase was attached. The following 8 ml. of eluate were collected and discarded. The remaining eluate was collected in a 250 ml. round bottomed flask and clearly marked "estrone." When 1 ml. of mobile phase remained on the surface of the column, a separatory funnel containing 60 ml. of ethylene dichloride-benzene (3/1) was connected to the column. From that moment the eluate was collected in a separate flask clearly marked "estradiol." The eluates for estrone and for estradiol were taken to dryness in vacuo on a water bath following the addition of a glass bead. These were considered to be impure estrone and estradiol fractions. The percolation rate of each column was checked with the desire that it be ten to twelve ml. per hour with 5 ml. of mobile phase above the celite column.

(7) Saponification of the Impure Estrone and Estradiol Fractions:

10 ml. of normal sodium hydroxide were added to each flask containing impure estrone or estradiol-17 beta and refluxed for thirty minutes. They

were cooled, acidified using 1.5 ml. of 12 N sulfuric acid and the solution transferred to 125 ml. separatory funnels. The flasks were rinsed twice with 10 cc. portions of benzene and the benzene transferred to the separatory funnels. The funnels were then shaken thirty times with sufficient vigor to insure extraction but not to produce emulsification. With separation of the lower layer, one drop was removed and the pH evaluated to assure its acidity. The remainder of the aqueous phase was then discarded and the benzene layer washed with 4 ml. of N Sodium carbonate. This was shaken and allowed to stand for five minutes and then subjected to shaking again. With the occurrence of a clear aqueous layer, it was removed. The additional shaking was required because of a complex formed between sulfuric acid and benzene requiring additional time to be disrupted by treatment with mild alkali. Water was added to the separatory funnel in a manner to insure washing of the ground glass stopper, inlet and walls of the funnel. The water was removed to the point where 1½ inches were left in the funnel. This was shaken. The aqueous phase was then removed. The water wash was again repeated. Repetition was performed to remove all traces of alkali. The benzene was then transferred quantitatively to Kober tubes which contained 50 mgms. of hydroquinone. The lip and inside of the separatory funnels were washed with benzene. The benzene was then evaporated on a boiling water bath under a jet of air. The estrone and estradiol extracts were then ready for colorimetric analysis. If high levels of estrone or estradiol were expected, for example in pregnancy urines or in recovery experiments, the benzene was transferred to 250 ml. round-bottomed flasks and the benzene removed in vacuo on a hot water bath following the addition of a glass bead.

To the 500 ml. round-bottomed flask containing the estriol residue and to the 250 ml. flask containing the higher levels of estrone and estradiol-17

beta residues, 3 ml. of pure alcohol were added in order to dissolve the residue. 2 ml. of the solution were pipetted to Kober tubes containing the hydroquinone. The ethanol was then evaporated by heating in a boiling water bath under a stream of filtered air.

(8) Color Development:

Standards were prepared by evaporating 0.1 ml. of ethanolic solutions of the three pure crystalline estrogens (0.1 ml. equalling 5 micrograms of the estrogen), in the presence of hydroquinone (50 mgms. plus or minus 5 mgms.) in Kober tubes. 3 blank tubes containing hydroquinone were also prepared. The appropriate color reagent was added from a burette to each tube (2.6 ml. for estriol, 3 ml. for estrone and 3 ml. for estradiol-17 beta.) All tubes were then heated in a boiling water bath for twenty minutes. The tubes were shaken laterally ten times during the third and again during the tenth minutes of heating. The tubes were then cooled in a bath of cold tap water and further hydroquinone added to each tube. To each estriol tube 0.7 ml. and to each estrone tube 0.3 ml. of distilled water was added. To each estradiol-17 beta tube 0.3 ml. of estradiol reagent were added. All tubes were then shaken and replaced in a boiling water bath for a further 15 minutes. They were again shaken during the third and tenth minutes of boiling in order to dissolve all hydroquinone. They were then cooled in a cold water bath. The optical densities of the solutions were read against the corresponding blanks in a Beckman DU spectrophotometer.

Estriol and estrone determinations were read at 480,512.5 and 545 millimicrons. Estradiol determinations were read at 480, 515, and 550 millimicrons. Corrected optical densities were obtained from the following equations: (28)

For estriol and estrone:

$$D_{\text{corrected}} = D512.5 - \tfrac{1}{2}(D_{480} + D_{545})$$

For estradiol:

$$D \text{ corrected} = D_{515} - \tfrac{1}{2}(D_{480} + D_{550})$$

The amount of estrogen in each fraction was determined from its corrected optical density and that of the appropriate standard by simple proportion or by referring the optical density to an appropriate standard graph.

To obtain the amount of estrogen in the entire 24 hour urine specimen, the estrogen in the aliquots used in the color reaction was multiplied by 7.5 for the determination of estriol and by 5 for the determination of estrone and estradiol-17 beta. These factors corrected for the dilution in the course of the determination. Alteration in the size of the aliquot required appropriate alteration in the correction factor.

(9) Precautions to be taken:

Many minor precautions were found necessary. All glassware was marked with a diamond pencil. Grease pencil was not permitted in the laboratory. Ground glass joints were permitted to contact glass only. All transfers to separatory funnels were accomplished through glass funnels. The precautions in handling the glassware were strict. All glassware was thoroughly rinsed in tap water followed by distilled water immediately following use. When they became noticibly dirty they were cleaned in chromic acid solution, washed in tap water and distilled water, soaked in crude ethyl alcohol and finally rinsed with tap and distilled water and then permitted to dry. All pipettes and Kober tubes were cleaned in chromic acid, water, alcohol, water and distilled water following each use.

All glassware used in an analysis of a specimen of high estrogen content was acid cleaned. Glassware having been soaked in acid was routinely immersed in alcohol in order to remove all traces of the chromic acid. Graduate cylinders used to measure reagents were marked in diamond pencil and used for that reagent only. Careful storage concerning dust and contamination was pertinent.

III. EXPERIMENTAL

A. Establishment of the Method:

(1) Standard Curves: To test the validity of the Kober Reaction and the stability of the reagents and standards, standard curves were determined for each of the three estrogens over a period of several weeks. The reagents were prepared as previously described. Pure crystalline estriol, estrone and estradiol-17 beta were obtained from Dr. W. S. Bauld, Montreal General Hospital, Montreal, Quebec. A stock standard was prepared by weighing 2.5 mgms. of the pure crystalline estrogen and diluting to 50 ml. with purified ethyl alcohol to give a final concentration of 5 micrograms/0.1 ml. These solutions were stored under refrigeration at 4° C. Appropriate amounts were taken to give concentrations of 2.5, 5, 7.5 and 10 micrograms of estrogens for purposes of color reactions. The optical densities were determined on a Beckman DU spectrophotometer against reagent blanks and at wavelengths previously outlined for estriol and estrone and estradiol-17 beta. The corrected optical densities were determined using Allen's correction formula. (28)

(2) Maximum Absorption Curves: As the corrected optical densities for standard material appeared to be approximately one-half of those shown by Bauld, the absorption spectrum of the final colored product for each estrogen was determined. Following full color development, the optical densities were plotted for each estrogen at various wavelengths, the absorptivity i.e. a(1%, 1 cm.) maximum wavelength, was calculated for each estrogen.

(3) Control of variable factors in Column Partition Chromatography: To avoid necessity for collection and analysis of each column eluate in small fractions, it was essential that the elution pattern be reproducible. To obtain reproducibility, several variable factors required control. (47)

(a) Column Parameters: Uniform column packing throughout the length of each column as well as from column to column was required. Accuracy in uniformity was determined by evaluation of the cross-sectional areas occupied by the inert phase (celite), the stationary phase and the mobile phase. These were designated A_I, A_s, A_1 respectively. A series of columns were packed until A_s/A_1 ratio was reproducible to within 5 percent. This was the maximum error allowable. The method used for estriol was as follows:

5 gms. of dried celite were weighed into a beaker of known weight. To this 5 mls. of stationary phase were added and mixed. 5 mls. of stationary phase was also added to the weighing bottle to permit calculation of the number of grams of the stationary phase equivalent to 5 mls. Mobile phase was then added to form a slurry and the weight of the total slurry determined. The column was then packed using the previously described packing technique. The weight of the slurry remaining in the beaker was determined. The difference was the weight of slurry packed into the column. This weight divided by the total weight multiplied by the weight of dry celite used gave the weight of the celite in the column. This weight divided by the density of celite permitted calculation of the volume of celite in the column. The volume divided by the length determined the cross-sectional of the chromatogram occupied by the inert phase celite i.e. A_I. Similarly the weight of stationary phase of the column was calculated and this weight was converted to volume. Subsequently the cross-sectional area occupied by stationary phase (A_s) was calculated.

Sudan IV, with a partition coefficient of zero in the system used, was added to the top of the column in mobile phase. The eluate was collected from the time the dye was added until it appeared at its maximum concentration.

in the eluate. This volume of eluate was then the retention volume (V_r) of the dye of K = 0. Butt's Equation (61) was then used.

This equation $K = \frac{V_r}{LA_s} - \frac{A_1}{A_s}$ can be arranged to $V_r = KLA_s + LA_1$ (47)

Because K = 0 then $V_r = LA_1$. From the known values of V_r and L, the cross sectional area occupied by the mobile phase (A_1) was calculated.

The percolation rate of all columns was noted in milliliters per hours.

K = Partition Coefficient i.e. $\frac{\text{Concentration of solute in stationary phase}}{\text{Concentration of solute in mobile phase.}}$

V_r = retention volume of the solute i.e. the volume of mobile phase passing through the column from the time of application of solute to column to appearance of concentration of solute in eluate.

L = Length of the chromatogram.

A_s = the cross-sectional area of the chromatogram occupied by stationary phase.

A_1 = the cross-sectional area of the chromatogram occupied by mobile phase.

The parameters of the estrone-estradiol-17 beta columns were accomplished in the same fashion other than the fact that 6.25 gms. of dry celite were weighed and packed into columns 12 cm. in length.

While the dye was passing through the column, it was carefully observed. Any loose packing or the presence of air bubbles would permit channeling of the chromatographic zone.

(b) Temperature Effect on Partition Coefficients: As partition coefficients and the mutual solubilities of stationary and mobile phases varied with temperature, (47) the partition coefficient of each of the three estrogens was determined. Equilibration of the solvents was accomplished in a temperature controlled room at the same temperature the chromatograms were to run. The columns were packed and the parameters determined. The pure estrogen was added

to the top of the columns in mobile phase. (3 x 1 ml.) The estrone and estradiol-17 beta were added together as they became separated on the same column. The collection of eluate in 2 ml. fractions was begun at the time of the first addition of the estrogen to the top of the column. As the last milliliter entered the columns, reservoirs were attached containing 30 ml. of mobile phase for estriol and 40 ml. mobile phase for estrone. When 1 ml. of mobile phase was left on the estrone column, a second reservoir containing 60 ml. of mobile phase for estradiol-17 beta was attached to the column. The collection of 2 ml. fractions of eluate was accomplished by means of a fraction collector. The solvent was removed from each fraction in vacuo. The residues were dissolved in alcohol and 2 ml. aliquots were added to Kober tubes and the color reaction determined in the usual fashion. The optical densities were determined for each fraction using Allen's correction equation. (28) From the volume of eluate containing the maximum concentration of the estrogen, the retention volumes of the columns were determined. Using Buttis equation, (61) the partition coefficients were calculated. The temperature of the room was noted at the beginning and at intervals during chromatography. An alkali concentration of 0.8 N was maintained throughout the experiments.

(4) Pure Estrogen added to the Column: The elution powers of the columns was determined by adding a known amount of pure estrogen and collecting the entire eluate in one flask. Solvents were prepared and allowed to equilibrate as previously described. Columns were packed in the usual fashion. 25 micrograms of each estrogen were added to the top of appropriate columns in their mobile phase. Collection of the eluate was begun immediately into 500 ml. round-bottomed flasks for estriol and into 250 ml. round-bottomed flasks for estrone and for estradiol-17 beta. The estrone and estradiol-17 beta were added to the same column, the reservoir and flasks being changed for estradiol as previously

described. The solvents were then removed in vacuo and the residue in the flasks dissolved in 3 ml. of pure ethanol. 1 ml. of this ethanol solution was added to the Kober tubes and a color reaction performed in the usual fashion.

(5) Estimation of Urinary Estrogens:

(a) Reproducibility and Recovery Studies:

Following the establishment by dye studies that the columns gave reproducible elution patterns and consistent A_s/A_1 ratios, the reproducibility of the duplicates and recoveries was done. 24 hour urines were collected in the special glass-stoppered bottles without preservatives and kept cool. The subjects were normal males and patients with estrogen levels presumed to be low. Urines were diluted to 2500 ml. volume and 500 ml. aliquots taken for hydrolysis.

Estrogens of known concentration were added to one of the hydrolyzates and the total estrogen content of the urine determined by the complete procedure described under Method. Simultaneous analysis was carried out on the remaining hydrolyzate to determine the endogenous content of estrogen. This value was used to correct the total in order to permit calculation of the recovery.

The remainder of the diluted urines were placed in the deep-freeze and kept frozen (59) until a later date when they were thawed and analyzed, again with recoveries to determine the reproducibility of the method. Any technical errors noted during the procedures were recorded.

(B) Clinical Study

1) Diurnal Rhythm: Information concerning the diurnal variation in excretion rates of many hormones and electrolytes has been well established. Reports of diurnal rhythm of estrogens were not discovered in the literature. A study of

a normal young unmarried female, a young married female, and a young pregnant multiparous female, were undertaken.

Twelve hour urines were collected in the following fashion:

On Subject I the normal young unmarried female, the first collections were taken on the eighth day of her menstrual cycle - the first specimen beginning at 7 a.m. and the night specimen beginning at 7 p.m. The second sample was collected on the fifteenth day of her menstrual cycle - the day specimen beginning at 10 a.m. and the night specimen at 10 p.m. The third sample was collected on the 19th day of her menstrual cycle - the day specimen beginning at 10 a.m. and the night specimen beginning at 10 p.m. The fourth sample was collected on the 27th day of her menstrual cycle - the day specimen beginning at 7 a.m. and the night specimen beginning at 7 p.m.

On Subject II the normal young married female, the first sample was collected on the 21st day of her menstrual cycle, the day specimen beginning at 8 a.m. and the night specimen beginning at 8 p.m. The second sample was collected on the 31st day of her menstrual cycle, the day specimen beginning at 8 a.m.and the night specimen at 8 p.m.

On Subject III the young pregnant multiparous female, a sample was collected about the 8th week of pregnancy, the day specimen beginning at 10 a.m. and the night specimen beginning at 10 p.m.

All samples were collected in special glass-stoppered bottles without preservative and appropriately labelled day and night. The volumes were measured and diluted to 1250 mls. The estrogen fractions of each sample were determined by the complete procedure described under method. Recovery analyses were done on the first two samples of Subject I and on the sample of Subject III. Duplicate analysis were done on all other samples. The results shown in Table 9 are the mean values, obtained from the duplicates.

IV. RESULTS

(A) Establishment of the Method:

(1) Standard Curves: A proportional relationship between the concentration of the estrogen and the corrected optical density was discovered. The data demonstrating this observation are shown in Table I. The linear relationship is demonstrated in Figure 3. The stability of the estrogen and reagents was discovered. This is also shown in Table I.

(2) Maximum Absorption Curves: The maximum absorption of estrone and estriol was shown to occur at wavelength 512.5 mu. and at 515 mu. for estradiol-17 beta. These data are disclosed in Table II and demonstrated in Figure 4. The absorptivity was approximately 1700 for estrone, 1400 for estradiol-17 beta and 1200 for estriol.

(3) Control of Variable Factors:

(a) Column Parameters: With experience and practice, adequate reproducibility of the estriol columns with A_s/A_1 ratio within 5% of the mean was accomplished. These data obtained from 17 columns are shown in Table III. Similarly the parameters of estrone - estradiol-17 beta columns were established. Table IV demonstrated these data. The initial values demonstrated (columns 1 to 9) outline the various results in the A_s/A_1 ratio obtained early in the experiment. The means calculated were based on data obtained from columns 10 to 22. These columns disclose consistent results obtained through practice.

(b) Temperature Effect on Partition Coefficient: The retention volumen and partition coefficients was found to vary inversely with the temperature of the chromatography room. This effect is disclosed in Table V.

(4) Pure Estrogen Added to the Columns: When pure estrogens were added to the columns the reproducibility of the columns and elution power was

shown on the duplicate analysis. This fact is shown in Table VI.

(5) Estimation of Urinary Estrogens:

(a) Reproducibility and Recovery Studies: The results of duplicate analysis are shown in Table VII. The first six estriols and the first five estrone and estradiol-17 beta were duplicates done on the same sample but on different days. The remainder are duplicate analysis done on the same sample at the same time. The results indicate that the method was reproducible.

The mean recoveries and their range are shown in Table VIII where 15 µg and 20 µg of pure estrogen were added to the hydrolyzates. The range of recoveries was noted to be lower than those reported (27) but the concentration of pure estrogen used was greater. They were calculated from the 500 ml. fraction not the total 2500 ml. volume.

(B) Clinical Study:

(1) Diurnal Rhythm: The results obtained for the three subjects are shown in Table IX. The results of the first two samples of Subject I are shown in Figure 5. It is noted that in the estriol determinations that a difference of 11.78 micrograms and 7.16 micrograms occurred. It was believed that contamination was present during the determination of the night specimen at which 11.78 micrograms was determined. This would lead to an erronously high value. In Subject III the difference of 24 micrograms and 14 micrograms occurred in the estriol determination. A loss of extract during the carbon dioxide treatment on the night specimen at which 14 micrograms was determined was believed to account for this lower value.

TABLE I

Corrected Optical Densities vs. Concentration.

Estrogen	S_1 (2.5 µg)	S_2 (5 µg)	S_3 (7.5 µg)	S_4 (10 µg)
Estriol	.0591	.1141	.1764	.2316
	.0532	.1096	.1713	.2174
	.0592	.1128	.1719	.2212
	.0512	.1096	.1684	.2144
	.0504	.1145	.1712	.2294
	.0564	.1101	.1661	.2192
	.0554	.1145	.1753	.2241
Mean	.0550	.1122	.1715	.2225
SD	± .004	± .0023	± .0057	± .0065
SE	± .0015	± .0008	± .0021	± .0025

Table I con't:

Estrogen	S_1 (2.5 µg)	S_2 (5 µg)	S_3 (7.5 µg)	S_4(10 µg)
Estrone	.0846	.1891	.2801	.3695
	.0935	.1878	.2887	.3652
	.0966	.1798	.2683	.3451
	.0937	.1837	.2744	.3487
	.0885	.1745	.2621	.3322
	.0900	.1740	.2620	.3405
	.0898	.1703	.2603	.350
Mean	.0909	.1799	.2708	.3502
SD	± .004	± .007	± .0107	± .013
SE	± .0015	± .0026	± .004	± .005

Table I con't:

Estrogen	S_1 (2.5 µg)	S_2 (5 µg)	S_3 (7.5 µg)	S_4 (10 µg)
Estradiol-17 b eta	.0649	.1399	.2120	.2832
	.0710	.1480	.2203	.2982
	.0685	.1455	.2188	.2872
	.0734	.1449	.2202	.2789
	.0800	.1480	.2265	.2819
	.0691	.1460	.2223	.2869
	.0709	.1486	.2257	.2858
Mean	.0711	.1458	.2208	.2860
SD	± .0057	± .004	± .0048	± .007
SE	± .0021	± .0015	± .0018	± .0026

TABLE II

The Relationship Between Absorbancy and Wavelength for the Final Colored Product Obtained from 10 µg. of pure Estrogen

Wavelength (mm)	Estriol (10 µg.)	Estrone(10 µg.)	Estradiol-17 beta (10 µg.)
450	.0655	.1037	.0809
460	.0955	.1427	.1079
470	.1397	.2076	.1487
480	.1939	.2882	.2076
490	.2403	.3670	.2676
500	.2882	.4410	.3280
510	.3400	.5300	.3980
512.5	.3520	.5650	.4140
515	.3470	.5610	.4260
517.5	.3400	.5570	.4230
520	.3280	.5530	.4200
530	.2100	.4090	.3230
540	.0862	.1990	.1580
545	.0434	.1149	.0915
550	.0177	.0655	.0505
560	0	.0188	.0144

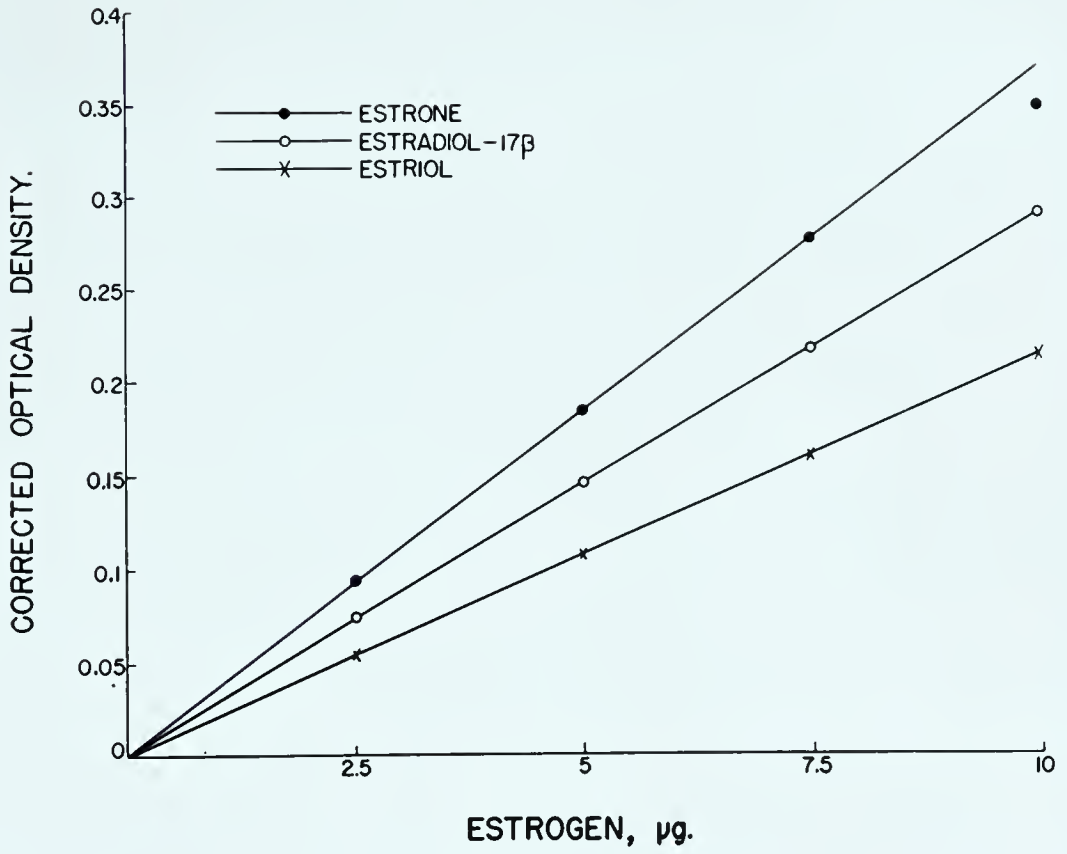


Figure 3. Corrected Optical Density vs. Concentration.

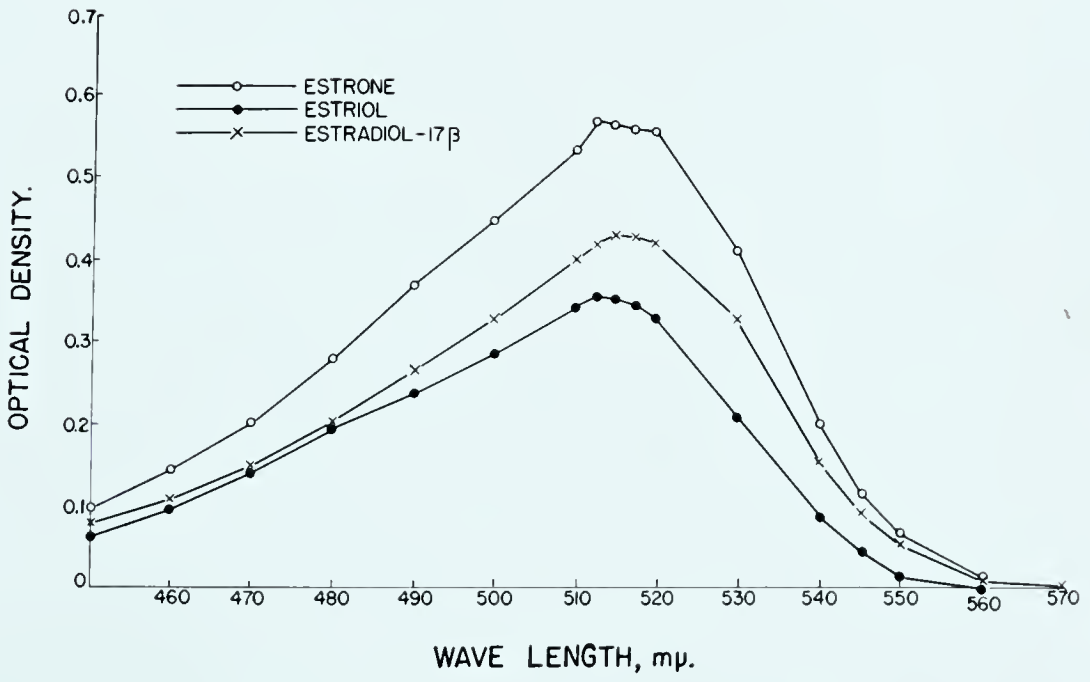


Figure 4. Maximum Absorption Curves.

TABLE III

ESTRIOL COLUMN PARAMETERS

Column No.	A_I CM^2	A_S CM^2	A_L CMS	A_S/A_L
1	.17	.34	.39	.87
2	.17	.35	.41	.85
3	.17	.35	.41	.85
4	.17	.31	.40	.78
5	.17	.35	.41	.85
6	.17	.34	.40	.85
7	.17	.33	.43	.77
8	.17	.35	.40	.89
9	.17	.34	.42	.81
10	.17	.33	.41	.80
11	.17	.35	.39	.89
12	.17	.34	.40	.85
13	.17	.35	.40	.87
14	.17	.34	.40	.85
15	.17	.35	.38	.92
16	.17	.35	.41	.85
17	.17	.33	.39	.84
18	.17	.33	.40	.82
19	.17	.36	.415	.87
Mean	.17 cm^2	.34 cm^2	.403 cm^2	.846 cm^2

TABLE IV

ESTRONE - ESTRADIOL COLUMN PARAMETERS

Column No.	A_I cm^2	A_S cm^2	A_L cm^2	A_S/A_l
1	.19	.35	.36	.97
2	.174	.32	.374	.85
3	.2	.37	.35	1.05
4	.19	.35	.366	.95
5	.19	.34	.36	.94
6	.195	.36	.35	1.03
7	.19	.35	.36	.95
8	.19	.36	.37	.97
9	.196	.365	.35	1.04
10	.181	.335	.39	.86
11	.18	.33	.43	.77
12	.18	.33	.40	.82
13	.18	.33	.40	.82
14	.18	.33	.39	.84
15	.18	.33	.40	.82
16	.18	.33	.43	.77
17	.17	.31	.44	.70
18	.17	.32	.40	.80
19	.17	.32	.43	.74
20	.17	.32	.41	.78
21	.174	.324	.413	.78
22	.18	.335	.40	.84
Mean	.176 cm^2	.326 cm^2	.41 cm^2	.795 cm^2

TABLE V

TEMPERATURE EFFECT

Estrogen	Temperature	Retention Volume	Partition Coefficient
Estriol	18.8° C	22	4.84
		22	4.94
		24	5.51
	19.1° C	24	5.32
		24	5.38
	28° C	20	4.94

Table V con't:

Estrogen	Temperature	Retention Volume	Partition Coefficient
Estrone	20° C	18	3.22
		22	4.55
		21	3.79
		21	3.96
	20-21° C	17.5	2.83
		19.75	3.67
	19.1° C	22	4.31
		24	4.76
	18.1° C	22	4.29
	17.8° C	26	5.35
		26	5.30

Table V con't:

Estrogen	Temperature	Retention Volume	Partition Coefficient
Estradiol-17 beta	20° C	64	14.08
		68	16.24
		64	13.47
		69	15.40
	20-22°	56.5	11.25
		60	13.25
	19.1°	56	12.43
		58	13.34
	18.1°	78	17.74
	17.8°	74	17.47
		74	17.32

TABLE VI

Recovery of 25 µg. of Estrogen from the Column

Estrogen	Amount Recovered	Deviation	% deviation from the mean
Estriol	23.85 µg 24.18 µg	± .165	.6%
Estrone	23.64 µg 24.45 µg	± .405	1.6%
Estradiol-17 beta	23.43 µg 23.43 µg	± 0	0%

TABLE VII

DUPLICATE ANALYSIS OF URINARY ESTROGENS

Estrogen	#1 µg	#2 µg	Deviation	% Deviation from Mean
Estriol	29.7	28.67	± .51	1.7
	36.6	44.7	± 4	10
	2.7	4.7	± 1	27
	2.4	4.5	± 1.05	30
	15.32	23.25*	± 3.96	20.5
	10.42	21.95*	± 5.77	35
	19.54	20.5	± .48	2.3
	31.65*	26.14	± 2.75	9.5
	13.39	13.55	± .08	.59
	10.8	10.8	± 0	0
	5.7	5.36	± .17	3
	12.97	12.15	± .41	3.2
	4769	4957	± 94	1.9
	6400	6828	± 214	3.2
	3744	3648	± 48	1.3
	5022	4870	± 76	1.54
	7658	7436	± 111	1.45

* Final Color after color reaction was brown.

Table VII con't:

Estrogen	#1 µg	#2 µg	Deviation	% Deviation from Mean
Estrone	6.5	9	± 1.25	16
	5.4	4.25	± .58	12
	9.37	3.5	± 2.935	45
	5.85	5.62	± .11	2
	14.6*	4.3	± 5.15	54
	3.49	4.31	± .41	10.5
	2.44	2.96	± .26	9.6
	2.48	2.11	± .185	8
	2.44	2.51	± .035	1
	2.44	2.78	± .17	6.5
	6.26	6.56	± .15	2.3
	5.70	5.17	± .265	4.8
	262	210	± 26	11
	318	335	± 8.5	2.6
	463	435	± 14	3.1
	262	343	± 41	13.5
	260	306	± 23	8.1

Table VII con't:

Estrogen	#1 µg	#2 µg	Deviation	% Deviation from mean
Estradiol-17 beta	5.5	4.25	± .635	13
	2.5	6.5	± 2	50
	8.25	3.75	± 2.25	37.5%
	3.37	3.25	± .06	1.8
	3.37	2.55	± .41	14
	3.37	4.05	± .34	9
	2.74	2.66	± .04	1.5
	1.54	1.73	± .1	6.1
	1.47	1.32	± .075	5.3
	1.28	1.01	± .135	11.7
	2.81	2.89	± .04	1.4
	2.33	2.40	± .035	1.4
	94	91	± 1.5	1.6
	54	69	± 7.5	12.2
	49.7	42	± 3.9	8.5

TABLE VIII

RECOVERY STUDIES

Addition of 20 µg. to the Hydrolyzate

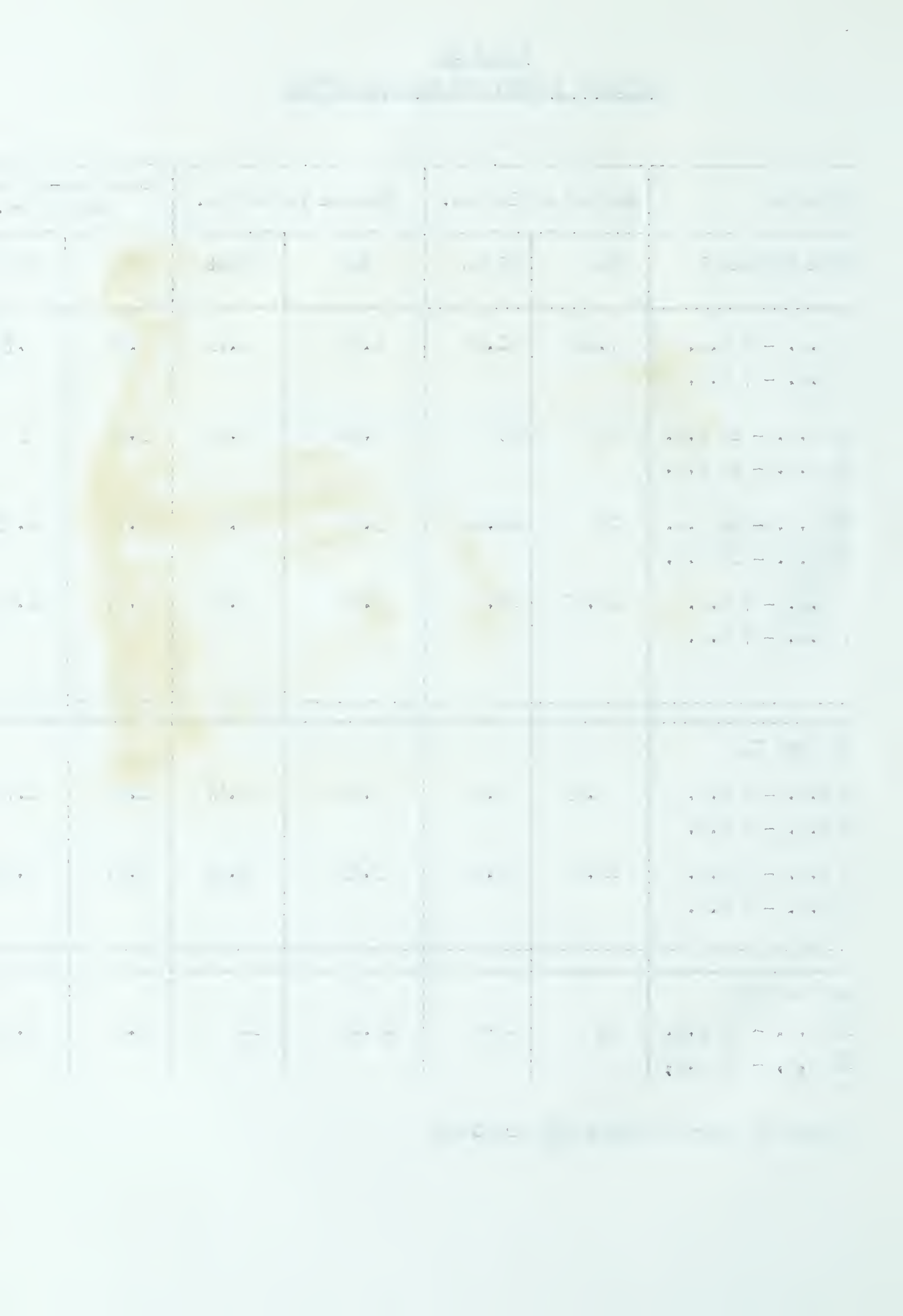
Estrogen	No. of Studies	Mean	Range
Estriol	5	71.9%	57% - 100%
Estrone	6	66.6%	58% - 72.5%
Estradiol-17 beta	6	61.5%	63% - 68.5%
Addition of 15 µg. to the Hydrolyzate			
Estriol	6	68%	54% - 84%
Estrone	6	73%	64% - 83%
Estradiol-17 beta	6	69.6%	60% - 80.5%

TABLE IX

ESTROGEN LEVELS FOR DIURNAL RHYTHM

Estrogen	Estriol µg/12 hrs.		Estrone µg/12 hrs.		Estradiol-17 beta µg/12 hrs.	
Time Subject I	Day	Night	Day	Night	Day	Night
7 a.m. - 7 p.m. 7 p.m. - 7 a.m.	7.16	11.78	1.84	1.73	.79	.97
10 a.m. - 10 p.m. 10 p.m. - 10 a.m.	19	19	4.24	6.18	3.75	3
10 a.m. - 10 p.m. 10 p.m. - 10 a.m.	20	26.14	3.49	2.7	2.7	1.63
7 a.m. - 7 p.m. 7 p.m. - 7 a.m.	13.47	10.8	2.29	2.06	1.39	1.14
Subject II						
8 p.m. - 8 a.m. 8 a.m. - 8 p.m.	5.53	4.13	2.61	2.47	1.54	1.76
8 p.m. - 8 a.m. 8 a.m. - 8 p.m.	12.4	12.56	5.43	6.41	2.37	2.85
Subject III						
10 a.m. - 10 p.m. 10 p.m. - 10 a.m.	24	14*	10.15	10	4.6	4.25

* Loss of extract during CO_2 treatment



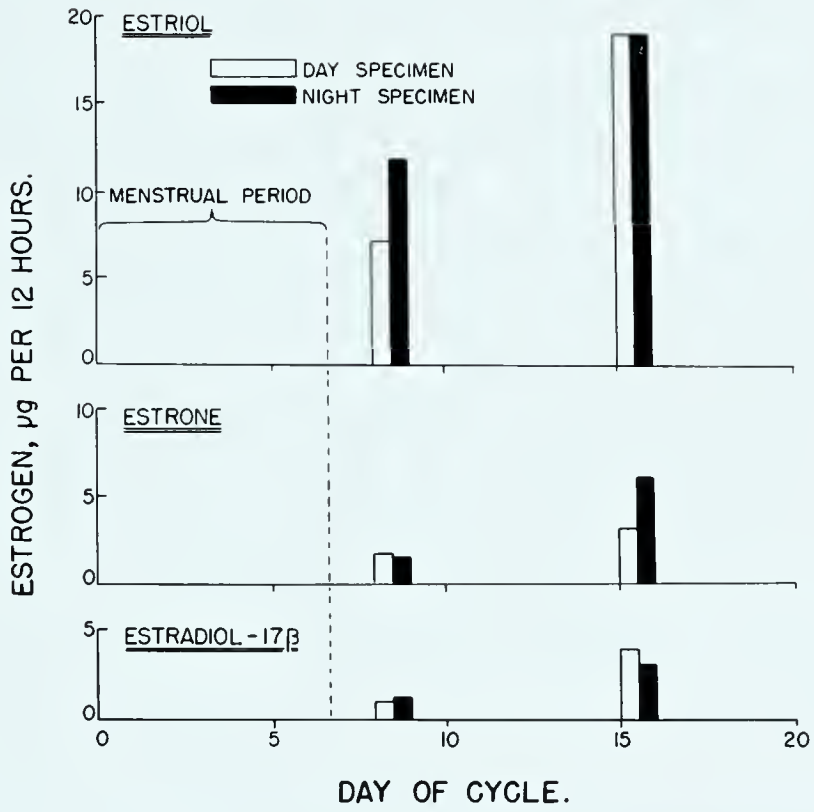


Figure 5. Day - Night Levels of Urinary Estrogen Excretion.

DISCUSSION

(A) Establishment of the Method:

(1) Standards and Maximum Absorption Curves: The corrected optical densities determined from varying the concentration of pure estrogen were practically identical over a period of several weeks. The values did not compare with those shown by Bauld (23) using the same concentration of the standard. This fact was found to be disturbing. Reagents and standards were freshly prepared and recalculation of the standards using Allen's correction formula (28) did not alter the values obtained in this laboratory. The value of the standards was found to be consistently $\frac{1}{2}$ of those shown by Bauld. A maximum absorption curve for each estrogen was therefore established. Using the same amount of standard estrogen, ten micrograms, the optical densities were plotted for each estrogen at different wavelengths. The maximum wavelengths were found to be the same as those published by Bauld. (23) The absorptivity as calculated by $E^{1\%}_{1\ cm.}$ i.e. the absorbance of one gram of material in a 1 cm. cell, was found to be exactly the same as reported for estrone and estradiol-17 beta and slightly lower for estriol. With the absorptivity being calculated to be practically the same as those reported, (23) incorrect use of Allen's correction formula might be suspected. However, no error could be found. Following personal communication with the author, an error in his article was elucidated. The corrected optical densities had not been divided by 2. When the data on Bauld's article were corrected the values were practically the same as those obtained in this laboratory.

The standards obey Beer's law over a suitable range of concentration.

The color reagent for estriol appeared to be less stable than the other color reagents. This reagent gradually altered from yellow to a brownish-tinged fluid. This change occurred with age. In preparing the reagent it was found that heating to the first tinge of yellow-green and then adding twenty grams of hydroquinone, followed by treatment in a boiling water bath for forty-five minutes, introduced greater stability than if heated to a definite yellow-green color.

The first stage of the color reaction is essentially a dehydration of estriol to estrone. The reducing agent hydroquinone is necessary to prevent the conversion of estriol to a product which would not undergo the second stage of the Kober reaction. (22)(23) The hydroquinone undergoes sulfonation decreasing its power of reduction therefore free hydroquinone is added before the color reaction.

The second stage is an oxidation reaction. (22)(23) Therefore nitrate and quinone are added to give oxidation products of hydroquinone (47) thereby standardizing the reagent. It may be postulated then, that excess heating of the aqueous acid solution in the preparation of the color reagent caused some of the oxidizing power of the solution to be destroyed. If this were so then the color reagent would have more reducing than oxidizing power and the final red color of the Kober reaction would therefore be lessened.

(1) Control of Variable Factors:

(a) Column Parameters: The technique of column packing was acquired following several weeks of experience. The first columns were packed to check percolation rates only. The solvents for estriol were used in practise runs. When percolation rates of 10 - 12 ml./hr. became reasonably consistent, Sudan IV was added to the column. The dye, as it progressed down

the column, revealed loose packing of celite near the walls and the presence of air bubbles. Channeling of the chromatographic zones would therefore result. Some workers (57) had recommended passing Carbon dioxide through the column before chromatography, but the danger of air bubbles and thus channeling would suggest that it should be avoided. The absence of excessive channeling was obtained by exerting a uniform pressure over the whole surface of the chromatogram as the segments were added. After fifteen months of packing columns, it has not been possible to pack more than a few that present an absolutely even front of dye. As the chromatography progresses a slight irregularity is usually seen.

Difficulty was encountered in calculating the parameters of the columns until the method as outlined under Experimental Section 3 (a) was derived. Using this method the ratio, A_s/A_l, was found to be consistent demonstrating the reproducibility of the columns.

In spite of experience in packing the estriol columns, difficulty was encountered when estrone-estradiol columns were attempted. The consistency of the slurry was different due to the higher interfacial tension. As shown in Table IV, the reproducibility of the columns was acquired only by experience. The A_s/A_l ratios of the first nine columns are noticably inconsistent.

(b) Temperature Effect on Partition Coefficient: All solvent equilibration, column packing and chromatography were carried out in a temperature controlled room. A temperature of 18° C plus or minus 0.5° was suggested in Bauld's method. (27) Unfortunately it was not always possible to establish this temperature. It was suggested (62) that maintainance of a steady temperature, irrespective of that temperature, was most

important. This was achieved. The retention volume and partition coefficient determined at various temperatures discloses the variation with alteration in temperature. The marked variation of estrone and estradiol partition coefficients may be noted. This variation suggested that the solubility of alkali in benzene plays an important part in determining the distribution of the estrogens. (58) With the disclosure of the value of temperature control, a separate small room was established in the University of Alberta Hospital utilizing a window air conditioner and improvised ventilation control. With this equipment, stable temperatures were achieved.

Estrone in the presence of urinary contaminants was found to be eluted more quickly than pure estrone, i.e. the retention volume was lower. It had been suggested (58) that these contaminants exert a displacement effect with aqueous alkali as the stationary phase. The elution of estriol was not affected by the presence of urinary contaminants. Because of the higher temperatures used than outlined in the Bauld Method and the presence of urinary contaminants, the collection of the eluate was begun earlier than was suggested under methods when the temperature was higher than 18° C.

(4) Estimation of Urinary Estrogens:

(a) Reproducibility and Recovery Studies: The sensitivity of the method was reported to be 5 - 10 micrograms/day for each estrogen with a possible error of plus or minus 15%. (27) This error increased to 20 to 25% at the 3 microgram level which is the limit of the sensitivity of the method. (47) In urines of low estrogen content there is a loss in precision. The fact that the subjects choosen were normal males and patients whose estrogen content was expected to be in low ranges, the discrepancies in duplicate analysis was considered acceptable. In most cases where a marked difference was noted

a cause could be demonstrated. Errors in techniques were the greatest contributing factor, such as leaking stopcocks, etc. This problem has been minimized by insuring the stopcocks and stoppers are not changed from separatory funnel to separatory funnel. Another difficulty was encountered in the complete removal of alkali from the extracts. The procedure finally used for washing was that described under methods and not as suggested in the method of Bauld. (27)

Bauld (27) in determining recoveries, added the pure estrogen to the hydrolyzate. However the amount he added was expressed in terms of the twenty-four hour volume. They were analysed according to the complete procedure described under method, with duplicate determinations for the endogenous estrogen content run at the same time. The value obtained was used to correct for the recovery. From his table of values (27) the addition of 25 µg of each estrogen calculated to twenty-five hours gave the best recovery. This amount of estrogen would be equivalent to 5 µg per 500 ml. of hydrolyzate.

When recovery experiments were attempted the amount of estrogen added was calculated to 500 ml. of hydrolyzate. In all cases then the amount added was 3 to 5 times greater than the amount Bauld added. Consequently the concentration was too great particularly in the case of estrone and estradiol-17 beta recoveries where the benzene eluates were evaporated to dryness in the Kober tubes. Because of the increased concentration there was an increase in optical density and therefore greater error.

The procedure was altered to reduce the concentration and therefore the optical density. The procedure adopted was to add the benzene eluates to round bottom flasks, evaporate off the benzene in vacuo and then dissolve the residue in purified alcohol, namely, the procedure for the estriol fraction. Aliquotes of this alcoholic solution was added to the

Kober tubes containing hydroquinone and evaporated to dryness. When this was done the recoveries calculated showed improvement.

From the recovery values shown (27) a concentration of 25 ug. of each estrogen calculated per 24 hr. volume or 5 ug per 500 ml. of hydrolyzate would give ideal optical densities and therefore maximum recoveries. This concentration was used once but unfortunately it was for the first recovery experiment. Lack of experience and poor technique gave very poor results. Further recovery experiments at this concentration continue.

In view of these considerations , recoveries shown in Table VIII were felt to be acceptable. The range is lower than those reported (27) but some were from experiments done before the procedure was altered.

The duplicate analysis of the first six estriols and the first five estrone and estradiol-17 beta of the urinary estrogens as shown in Table VII were run on different days. This was done to permit evaluation of recoveries and to suit available space and equipment. The remainder of the values shown were duplicate samples done at the same time. Also shown in Table VII is the present deviation from the mean for each set of duplicates. The precision of the method is less at lower values. This is shown by the greater percent deviation for low values than for high values. With reference to the duplicate analysis shown by Bauld (27) where estrogen levels from 0 - 4 µg/24 hr., the % of variation was 0 - 38. Levels from 4 - 16 µg/24 hr. the % of variation was 0 - 19 and levels from 16 - 25 µg/24 hr. the % of variation was 0 - 10. It was felt that the duplicate analysis obtained for urinary estrogens were acceptable.

When duplicate analysis were done to show the reproducibility and elution power of the columns the results obtained, Table VI, show an error of approximately 5% in recovery. The results as duplicates showed a small

percent deviation from the mean. Therefore the columns were also reproducible.

The complete validity of the method has not been established as yet in this area. The length of time required for each determination and the few samples that could be run at one time has limited the number of determinations done to date. Therefore complete statistical analysis could not be carried out. It was felt that the method for urinary estrogens was adequately established.

(B) Clinical Study:

1) Diurnal Rhythm

The studies as shown in Table IX failed to disclose evidence of a diurnal rhythm in either estrone or estradiol-17 beta urinary excretion. The slight variations observed fall within the limit of error. The results of the estriol analyses were less conclusive. They presented occasionally somewhat larger variations which however showed no consistent trend. It is noted in Figure 5 a higher level of estriol was excreted in the night sample. It is felt that this was due to a technical error as the color reaction was brownish and murky suggesting contamination. This eluate had been collected in a flask originally used for estradiol-17 beta and was pitted from previous saponification. A recovery done on this specimen yielded a corrected optical density similar to that for the day specimen.

The lower result noted in Table IX for the estriol fraction on the night specimen of Subject III was believed to be due to a loss of estriol alkali solution during the carbon dioxide treatment.

The values obtained for Subject I show the familiar pattern of higher values at ovulation and at the luteal phase and lower values directly pre and post menstruation.

From this limited study no conclusion can be drawn about diurnal rhythm for the excretion of urinary estrogens. The study is being continued.

SUMMARY

1. A detailed procedure for the extraction, separation and purification of estriol, estrone and estradiol-17 beta in urine was evaluated and established.

2. The method involved: acid hydrolysis, ether extraction, separation of estriol from estrone and estradiol-17 beta by distribution between benzene and water, purification of estriol by saponification and column partition chromatography; separation and purification of estrone and estradiol-17 beta by column partition chromatography and saponification. The purified extracts were determined colorimetrically by an improved Kober reaction using Allen's correction formula (28) to correct optical densities for non-estrogenic material.

3. Subjects chosen for duplicate analysis and recovery experiments had low estrogen levels creating maximal discrepancy in results.

4. The standard and maximum correction curves were found to be identical with those reported.

5. Column parameters, effect of temperature on partition coefficients were investigated.

6. A clinical study evaluating the rate of urinary estrogen excretion with a view to assessing the possible existence of diurnal rhythm has been commenced. The evidence available to date suggests that a diurnal rhythm has not occurred in the normal young menstruating females, nor in a normal multipara pregnant female in either estrone or estradiol-17 beta urinary excretion. The results were less conclusive for the estriol fraction. This study is being continued for further evidence.

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